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January, 1941

Research Bulletin 285

Classification of the Organisms Important in Dairy Products

III. *Pseudomonas putrefaciens*

BY H. F. LONG AND B. W. HAMMER

AGRICULTURAL EXPERIMENT STATION
IOWA STATE COLLEGE OF AGRICULTURE
AND MECHANIC ARTS

DAIRY INDUSTRY SECTION

AMES, IOWA

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SUMMARY

A special gelatin agar containing an iron salt is very useful in the isolation of *Ps. putrefaciens* because of the luxuriant growth and high color production with it. Even on this medium, however, isolated cells may not initiate growth readily. With certain materials enrichment in litmus milk at 3°C., followed by smearing on the special medium, aids in isolating the organism, while with other materials direct smears are more frequently successful. In attempting isolations from butter, the serum is more satisfactory than the butter itself, and enrichment in laboratory churnings of butter (as well as in litmus milk) held at 3°C. may be helpful.

Ps. putrefaciens is widely distributed in certain dairy products, in water (streams, lakes, roadside pools, creamery supplies, etc.), on dairy plant floors, in dairy plant sewers and in dairy plant equipment.

Ps. putrefaciens is rather easily destroyed by heat, acid and salt. In butter the distribution of the salt, as well as the total content, is important from the standpoint of preventing growth of the organism.

There are variations between cultures of *Ps. putrefaciens*, but these do not appear to justify varietal designations.

The outstanding characters of *Ps. putrefaciens* from the standpoint of identification are action on litmus milk, morphology, phosphatase production and action on butter.

Classification of the Organisms Important in Dairy Products

III. *Pseudomonas putrefaciens*¹

BY H. F. LONG AND B. W. HAMMER

Development of a putrid or cheesy condition is one of the very serious types of bacterial deterioration in butter. While various species of bacteria undoubtedly can bring about this general type of change, the one of most importance appears to be *Pseudomonas putrefaciens*. It originally was isolated at the Iowa Agricultural Experiment Station and tentatively designated *Achromobacter putrefaciens* (3); additional work has indicated that it belongs to the genus *Pseudomonas*, and the designation *Ps. putrefaciens* is now employed.

Until recently, only a few isolations of *Ps. putrefaciens* had been reported. While the organism grows rapidly on various media after development once begins, it frequently fails to initiate growth so that isolations have been difficult. Numerous samples of putrid butter have been studied in various laboratories without isolating an organism capable of reproducing the original defect, and it is probable that in many of these *Ps. putrefaciens* was involved.

Because of the importance of the putrid defect in butter and the frequency with which *Ps. putrefaciens* is responsible, additional studies on the organism are detailed herein.

HISTORICAL

In 1931, Derby and Hammer (3) reported the isolation of an organism, which they named *Achromobacter putrefaciens*², from a number of samples of butter showing surface taint. The organism was not obtained consistently from this type of butter, although enrichment and various other procedures were employed. It was considered to produce the typical surface taint defect.

Cullity and Griffin (2) recorded a number of investigations at plants experiencing outbreaks of rabbit in Australian butter, and in each the defect eventually was traced to the factory water; new water sources eliminated the trouble. Apparently, *Achromo-*

¹ Projects 114 and 119 of the Iowa Agricultural Experiment Station.

² Throughout the historical section, the original genus name is employed to conform with the names in the various publications.

bacter putrefaciens was isolated from two water supplies, although the authors stated that there appeared to be some variation in the characters of the organisms.

Cullity and Griffin (2) also reported that Loftus Hills, Scharp and Searle isolated rabbit organisms from factory water supplies, churns and raw and pasteurized cream; water was considered to be the natural habitat. The organisms were similar to *Achromobacter putrefaciens* (3).

Claydon and Hammer (1) studied samples of commercial putrid butter and found that with few exceptions *Achromobacter putrefaciens* was the only organism obtained which was capable of reproducing the typical defect when inoculated into cream and the cream churned. The organism was not isolated by plating the samples, and the predominating species on plates poured with putrid butter did not reproduce the defect. By special procedures, *Achromobacter putrefaciens* was obtained from 41 (70.7 percent) of 58 commercial samples showing the typical putrid defect, and 6 (10.3 percent) yielded an apparent variant form; 3 (5.2 percent) yielded other organisms producing objectionable odors which were not typical of the putrid defect, while from 8 (13.8 percent) an organism causing an objectionable odor could not be isolated. It was noted that *Achromobacter putrefaciens* did not readily initiate growth on media, although it developed rapidly when growth had once begun. In experimental butter *Achromobacter putrefaciens* decreased in numbers as the butter aged, and became increasingly difficult to regain.

Canadian work, in which *Achromobacter putrefaciens* was isolated from putrid butter and also from water supplies of plants experiencing the defect, was cited by Claydon and Hammer (1).

Shadwick (7) obtained several cultures of *Achromobacter putrefaciens* from equipment in plants having difficulty with putrid butter.

EXPERIMENTAL

DEVELOPMENT OF AN ISOLATION TECHNIC FOR *Ps. putrefaciens*

The difficulty with which most cultures of *Ps. putrefaciens* initiate growth on agar and the presence in butter of other species which grow well on common laboratory media, complicate isolation of the organism from putrid butter. Usually, *Ps. putrefaciens* cannot be isolated directly from defective material, and enrichment procedures must be employed. Even with such

a precaution, it often is difficult or impossible to isolate the organism and, accordingly, various isolation methods were investigated.

The early isolations of *Ps. putrefaciens* were made on beef infusion agar (3). With a heavy inoculation on this medium, the organism usually develops rather well. However, with small inoculations, as generally is the case in attempting to isolate the organism from the mixture of species in putrid butter, growth commonly is uncertain, and even well-developed colonies are not readily detected. Attempts were made to promote growth of *Ps. putrefaciens* by adding various materials to beef infusion agar, growth being tested by streaking loops of various dilutions of different cultures on prepared plates and observing luxuriance of growth as well as the highest dilutions yielding growth.

EFFECT OF VARIOUS CHEMICAL COMPOUNDS

Growth of *Ps. putrefaciens* on beef infusion agar was not improved by addition of various amounts of the amino acids, alanine, asparagine, cysteine, cystine, glycine and tryptophane, or the surface tension depressants sodium oleate and sodium ricinoleate, or the miscellaneous compounds disodium phenyl phosphate, lecithin and sodium acetate.

Addition of the oxidizing materials, potassium permanganate, hydrogen peroxide and potassium persulfate retarded growth of *Ps. putrefaciens* when used in relatively large amounts and did not aid growth in small amounts. However, addition of certain reducing compounds was of some value, sodium thiosulfate apparently being best suited to the organism. When added to beef infusion agar in a concentration of 1 or 2 percent at the time of preparing plates, this material rather consistently favored initial growth of certain strains of *Ps. putrefaciens*. Although growth could be obtained from somewhat higher dilutions of some of the cultures by the use of sodium thiosulfate, it was not more vigorous, luxuriant or conspicuous than without the chemical, and the isolation procedure was not simplified. The reducing compounds, hydrogen sulfide, hydroquinone, n-propyl mercaptan, sodium sulfide and thioglycolic acid were not particularly effective.

Recent reports indicate that certain vitamins and plant hormones may increase multiplication of certain organisms. A few of these compounds and other materials having some chemical relationship to them were tested for their ability to stimulate growth of *Ps. putrefaciens*. The compounds investigated were α -naphthaleneacetic acid, benzoic acid, γ -(indole-3)-n-butyric acid, hydrocinnamic acid, indole-3-acetic acid, nicotinic acid, phenylacetic acid, riboflavin, thiamin and 1,2,5,6-dibenzanthra-

cene. With the exception of benzoic acid, which had a questionably beneficial effect, these chemicals did not aid growth.

EFFECT OF INCUBATION CONDITIONS

Incubating plates inoculated with *Ps. putrefaciens* in atmospheres of hydrogen sulfide, carbon dioxide, nitrogen or one of these gases mixed with various amounts of air did not stimulate growth. Incubation in a bell jar in the presence of slices of potato was not beneficial.

EFFECT OF MEDIA

Since addition of certain chemicals to beef infusion agar and incubation in atmospheres of different gases did not yield the desired results, various media were compared with beef infusion agar. These included liver, beet, cabbage and carrot infusion agars; tomato juice, beef extract, tryptone glucose skimmilk, milk digest, malt, potato dextrose, peptone iron, tryptone iron and North's gelatin agars; and egg glycerol medium. In addition, agar was added to skimmilk, cream and butter in sufficient concentrations to make solid media. A cell-free filtrate of a culture of *Ps. putrefaciens* grown in beef infusion broth also was added to beef infusion agar. The pH in each instance approximated 6.8, which is a satisfactory level for the species.

The media which were definitely superior to beef infusion agar in supporting growth of *Ps. putrefaciens* were egg glycerol medium and North's gelatin, peptone iron and tryptone iron agars. Although egg glycerol medium supported growth very well, it was so opaque that colonies were difficult to detect; consequently, the medium was not satisfactory for isolation purposes. Peptone iron agar was slightly better than tryptone iron agar and with addition of 4 percent gelatin was much superior to North's gelatin and beef infusion agars. This special gelatin agar has the following composition:

| | |
|-------------------------|---------------|
| Gelatin | 4.0 percent |
| Proteose peptone | 2.0 percent |
| Dipotassium phosphate | 0.1 percent |
| Ferrie ammonium citrate | 0.05 percent |
| Agar | 1.5 percent |
| Water to make | 100.0 percent |

On this medium *Ps. putrefaciens* develops readily, and after several days colonies are fairly large, raised and brown to reddish brown or pink; even with it, however, growth may not occur when the numbers of organisms in the inoculating material are small. Color production is much more intense than on the other media and is especially useful in isolation studies because *Ps.*

putrefaciens colonies often can be detected in an area heavily seeded with other species. Better results are obtained when plates are smeared than when poured in the usual way (1,4). The organism apparently finds difficulty in establishing itself on agar even under the best conditions and is more commonly obtained with the smear technic, in which several cells may be left in a clump, than with the plating method, in which the cells are more widely distributed. In examination of butter, better results have been obtained with the serum than with butter direct; presumably, the greater concentration of the cells is desirable.

EFFECT OF ENRICHMENT METHODS

Derby and Hammer (3) inoculated defective butter into litmus milk and incubated at 5°C.; although rapidly-reducing, acid-forming organisms often interfered with the isolations, five cultures of *Ps. putrefaciens* were obtained. Claydon and Hammer (1) suggested a procedure, described as relatively successful, which consisted of inoculating defective butter into pasteurized cream, holding overnight at 10°C. and churning. The resulting butter was divided and held at 21° and 5°C.; on development of the defect, the butter was smeared on beef infusion agar. Second or even third-generation churnings sometimes were of value. Enrichment of the serum of defective butter in litmus milk at approximately 5°C. also was suggested.

Various enrichment procedures were investigated in considerable detail. Addition of the serum of defective butter to litmus milk with holding at 5°C. was of some value, but on extended incubation acid formers almost always predominated. Incubation at temperatures higher than 5°C. was of even less value. Enrichment through experimental churnings was useful, but when *Ps. putrefaciens* was not recovered after relatively short holding periods at either 21° or 5°C., it was rarely isolated on longer holding; presumably, acid-forming organisms interfered with this procedure as they did with enrichment in litmus milk.

In an attempt to keep down acid-forming organisms during enrichment in milk at 5° or 10°C., crystal violet (1 to 5000) was added to the milk. The dye kept down the acid-forming cocci, but *Aerobacter* species often dominated the flora, and various *Pseudomonas* species developed rather well. A few isolations were made with the technic, but it was not especially useful. Substitution of Nile blue sulfate for crystal violet was not of value.

Enrichment in litmus milk at 3°C., rather than 5°C., often aided in isolating *Ps. putrefaciens* from putrid butter, water, soil and swabs smeared on floors, equipment, etc. Frequently,

the organism was isolated in this way when it was not obtained by direct smears or by other enrichment methods. In certain cases addition of 1 percent sodium thiosulfate to the milk at the time of inoculation was of value, whereas in other cases it was not helpful; probably the requirements of the particular strain of *Ps. putrefaciens* in the sample were the determining factor. With putrid butter the amount of serum usually added to 10 ml. of milk was 1 drop to 0.1 ml.; with a few samples, however, *Ps. putrefaciens* was not obtained with such an inoculation but was isolated when 1 to 2 ml. of serum was added to 10 ml. of milk. Enrichment for more than 10 days usually was required, and up to 30 days often was necessary. With some samples, carrying the material through several transfers in litmus milk was an aid in isolation. Commonly, isolation was not attempted until some reduction of the litmus occurred; however, reduction did not always indicate the presence of *Ps. putrefaciens*, since certain other species reduced milk at 3°C.

With some materials use of low-temperature enrichment in litmus milk was not as effective as direct smears. In the examination of one lot of samples from creamery equipment, a comparison was made of the direct smear and enrichment methods. *Ps. putrefaciens* was isolated from 13 of the 66 samples by direct smears. After enrichment in litmus milk for 2 weeks at 3°C., it was found in only 6 of the 13 samples from which it was obtained originally but was isolated from three additional samples. After enrichment for 4 weeks, the organism was found in only 2 of the 13 samples from which it was isolated originally and in one of the three samples from which it was obtained after enriching 2 weeks. Practically all the plates smeared with the enriched material showed profuse growth, and it appeared that extensive multiplication had taken place; presumably, in certain of the samples *Ps. putrefaciens* was outgrown by other species.

SUGGESTED ISOLATION PROCEDURE

In attempting the isolation of *Ps. putrefaciens* the special gelatin agar is advisable because of the more luxuriant growth and high color production with it. Direct smears of the material should be made, since with certain floras the chances of obtaining the organism at once are better than after enrichment. This is particularly true when other species capable of rapid growth at low temperatures are present. At the time direct smears are made, various amounts of the material can be added to litmus milk for incubation at 3°C. To delay growth of acid-forming species, the milk should be iced before inoculation and kept iced until placed at 3°C. On removal of the enriched material for smearing, it should be kept iced if the enrichment procedure is

to be continued. Usually, isolation from enriched material is not attempted until reduction of the litmus occurs, but it may be advisable to make smears before this in order to limit competition from other species.

With some materials, especially putrid butter, it is helpful to make experimental churnings, the purposes being to determine whether the material is capable of reproducing the putrid condition and to provide fresh material for isolation. Apparently, the organism dies off rather rapidly in salted butter at relatively high temperatures.

Examination of plates for *Ps. putrefaciens* colonies should be carried out under a good light. Usually colonies can be detected with the unaided eye, but often a low-power binocular is helpful, especially when the colonies are small and the plates crowded. In general, any colony having a brown to reddish brown or pink color and a granular appearance should be investigated. Since colonies of *Ps. putrefaciens* commonly develop in areas heavily seeded with other species, picking directly into litmus milk often yields seriously contaminated cultures, and it is advisable to begin purification immediately by smearing material from the colony on the special medium. Figure 1 shows a plate in which *Ps. putrefaciens* colonies are in areas heavily overgrown with other species; the plate was smeared directly with rotted wood from a churn.

DISTRIBUTION OF *Ps. putrefaciens*

Various materials, from different points in Iowa and from other states, have been examined for *Ps. putrefaciens*.

Ps. putrefaciens IN DAIRY PRODUCTS

MILK AND CREAM

Ps. putrefaciens was not obtained by direct isolation from milk or cream. With enrichment, it was obtained from 3 of 17 samples of raw milk and two of four samples of pasteurized milk; presumably *Ps. putrefaciens* gained entrance to the pasteurized milk after heating, since it has a low thermal resistance. By enrichment, *Ps. putrefaciens* was isolated from two of six samples of raw sweet cream; it was not obtained from several samples of sour cream.

BUTTER

Many samples of putrid salted butter were examined over a period of years in studies on the cause of the defect. *Ps. putrefaciens* was isolated from them only rarely by direct smears on

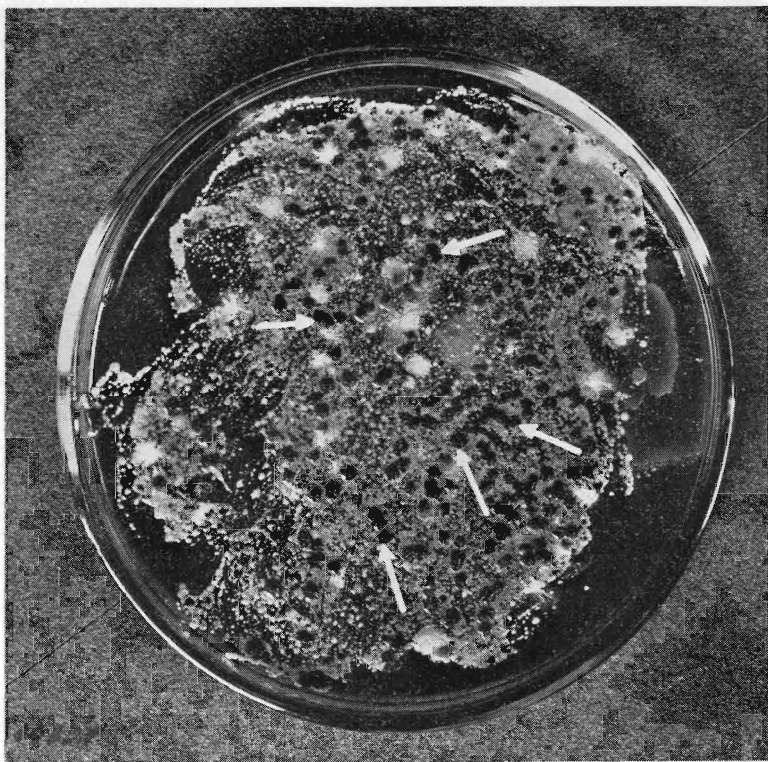


Fig. 1. Plate showing *Ps. putrefaciens* in areas heavily overgrown with other species; the plate of special gelatin agar was smeared directly with rotted wood from a churn.

beef infusion agar but was obtained from a number by smearing on beef infusion agar after enrichment in litmus milk or in experimental butter at approximately 5°C. Since this medium and enrichment temperature are not particularly satisfactory for isolating *Ps. putrefaciens*, the organism probably was in various samples from which it was not recovered. The more recent studies, in which serum from putrid butter was smeared on the special gelatin agar and also was added to litmus milk for enrichment at 3°C., have recovered *Ps. putrefaciens* from many samples of typically putrid salted butter. In some cases the organism was isolated directly, but usually enrichment was necessary.

Normal salted butter also yielded *Ps. putrefaciens*. In the examination of 19 samples it was obtained from two by enrichment but not by direct smears. One of the samples yielding

Ps. putrefaciens also was subjected to keeping-quality tests at 21°C. and did not show deterioration even on long incubation. Apparently, the presence of the organism in butter is not enough to give the putrid defect; fairly large numbers may be necessary, and conditions in the butter must allow growth (5).

Numerous attempts to isolate *Ps. putrefaciens* from highly ripened unsalted butter, some of which was defective and some normal, regularly failed. This would be expected on the basis of the high acid content of such butter.

Ps. putrefaciens IN MATERIALS OTHER THAN DAIRY PRODUCTS

WATER AND SOIL

With *Ps. putrefaciens* in raw milk supplies, sources of the organism on farms would be expected and, by enrichment in litmus milk, the organism was obtained from water and soil. Of 48 samples of stream and lake water collected in California, Idaho, Illinois, Indiana, Iowa, Kansas, Kentucky, Maryland, Minnesota, Missouri, South Dakota and Wisconsin, 40 yielded *Ps. putrefaciens*; of six samples of roadside water from Illinois, Indiana and Iowa, four yielded the organism. *Ps. putrefaciens* was recovered from one sample of moist soil but not from three samples of dry soil; the four samples were from Iowa. The organism also was isolated from water being used to wash the flank of a cow.

CREAMERY WATER SUPPLIES

Various investigators have emphasized the importance of creamery water supplies as a source of organisms causing spoilage in butter. In the examination of samples of water mailed from 29 creameries in Indiana, Iowa, Kansas, Minnesota, Nebraska, North Dakota, Oklahoma, South Dakota and Texas, *Ps. putrefaciens* was recovered after enrichment from the supplies of 14. Eight of the 29 creameries reported difficulty with the keeping qualities of their butter and submitted water samples because the water was under suspicion; *Ps. putrefaciens* was recovered from five of these eight plants. With the remaining 21 creameries, no information was obtained on the keeping qualities of the butter. In general, water yielding the organism came from private wells, although in a few cases city water supplies were involved.

CREAMERY FLOORS, SEWERS AND EQUIPMENT

In the examination of a surface for *Ps. putrefaciens*, a sterile, moist, cotton swab was smeared over the desired area and then

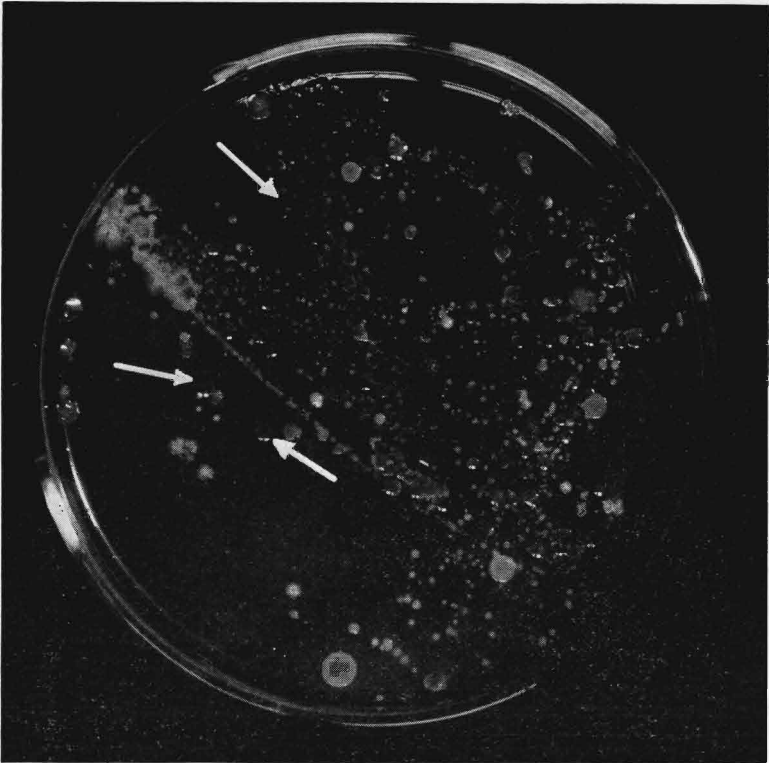


Fig. 2. Plate showing *Ps. putrefaciens* as the predominant species; the plate of special gelatin agar was smeared directly with material obtained from a joint in the end of a churn.

placed in litmus milk for enrichment at 3°C. *Ps. putrefaciens* was isolated readily from floors and sewers, where it might have been deposited from soil, cream or water. It was present in cream-receiving rooms, in churn rooms and in plants handling milk and cheese as well as in those handling butter. The organism was especially prevalent in areas on floors where moisture tended to remain for long periods. Other sources were a cream-receiving vat and the top of a cream can. The organism was obtained from various parts of three butter printers, including the box, auger, gear and belt of one in a plant manufacturing butter which sometimes became putrid. It could have been infected by the butter, since the butter contained the organism before it reached the printer. Swabs from pipe lines, pumps, holding vats and churns failed to yield the organism.

In addition to the churns examined with swabs, four were

subjected to more detailed examination by obtaining material from around bolt heads, between staves, at the junction of staves and ends, etc. Three of the churns were from plants experiencing the putrid defect; 83 samples were collected from these churns, and isolations were made directly from 17 and after enrichment from six additional. The fourth churn was from a plant having no difficulty with putrid butter, and *Ps. putrefaciens* was not obtained from it, although a variety of organisms was isolated.

In certain materials from plant equipment, much heavier concentrations of *Ps. putrefaciens* were encountered than in other sources investigated. Various plates smeared directly with such materials contained many colonies of *Ps. putrefaciens*, and in a few of them the organism predominated. Figure 2 shows a plate in which *Ps. putrefaciens* colonies predominated; the plate was smeared with material obtained from a joint in the end of a churn.

SPECIAL STUDIES ON *Ps. putrefaciens*

HEAT RESISTANCE

Previous investigators (1, 3) have emphasized the low thermal resistance of *Ps. putrefaciens*. However, continued spoilage of butter from various plants attempting to properly control the manufacturing operations made additional heat tests advisable.

In each trial cultures of *Ps. putrefaciens* were used which showed considerable variation from the standpoints of origin, action on litmus milk and period carried on artificial media. One ml. of a 1-day milk culture (grown at 21°C.) of each strain was added to 10 ml. of milk, and 2-ml. portions of each mixture were sealed in glass tubes. Similar tubes were prepared with a 7-day culture of each strain. The tubes were exposed in a water bath at 61.7°C. for different periods. The cultures used in the various trials regularly survived a 0.5-minute exposure, while none except the 7-day culture of one strain and the 1-day and 7-day cultures of another strain survived 1 minute, and these were killed in 2 minutes.

In several trials the milk suspensions of young and old cultures of the organisms were acidified to about 0.5 percent acid. After holding 1.5 hours, the material was neutralized, and heat tests were conducted in the usual manner. The treatment had no effect on the heat resistance of the organism.

The results agree with previous reports (1, 3) in indicating that *Ps. putrefaciens* is very susceptible to heat under the ordinary conditions and that it is unlikely the organism could survive either the flash or holding method of pasteurization.

ACID RESISTANCE

Acid resistance of *Ps. putrefaciens* was investigated by adding various amounts of lactic acid to 100-ml. lots of sterile skim milk and inoculating each with 0.1 ml. of a 2-day milk culture (grown at 21°C.) of the organism to be tested. The pH of each mixture was determined at once, and survival and growth of the organisms were determined at intervals by inoculating 0.1 ml. of the mixture into litmus milk and also by smearing 0.1 ml. on the surface of the special medium.

There was general agreement in growth on the agar and in litmus milk, although in several instances growth was obtained with the former but not the latter. Lactic acid had a pronounced destructive action when present in sufficient concentrations. At a pH of approximately 5.3, *Ps. putrefaciens* survived only a relatively short time, and usually no growth was obtained on agar or in milk after 48 hours. With pH values appreciably above 5.3, the organism multiplied in the acidified milk, and with values below 5.3, it was killed in less than 48 hours. In one lot of milk acidified to pH 4.9, the organism was killed in 8 hours.

The data are in agreement with those of Claydon and Hammer (1) who noted that butter churned from cream inoculated with *Ps. putrefaciens* developed the putrid defect only to a slight extent when the cream was previously acidified to pH 5.2 and not at all when the cream was acidified to pH 4.5. With higher pH values the defect was conspicuous. The sensitivity of *Ps. putrefaciens* to acid indicates that the use of butter culture in making butter should have a protective action as far as the putrid defect is concerned. It also suggests that *Ps. putrefaciens* should be destroyed in cream or butter containing considerable acid. In the examination of various dairy products, sour cream and highly ripened unsalted butter failed to yield the organism.

SALT RESISTANCE

The salt resistance of 15 representative cultures of *Ps. putrefaciens* was tested in litmus milk. The concentrations of salt included were 4, 6, 8 and 10 percent, and incubation was at 21°C.

In milk containing 4 percent salt, all the cultures grew; with 6 percent salt, only six of the cultures grew, and these grew much more slowly than at the lower concentration; with 8 percent salt, one culture showed slight growth after 3 weeks; and with 10 percent salt none of the cultures developed. The check cultures, which included a butter culture, *Pseudomonas fluorescens* and *Aerobacter aerogenes*, grew in milk containing 4 percent salt but not in milk with 6 percent salt.

Since the brine concentration in butter containing 15 percent

moisture and 1.5 percent salt averages 10 percent, the amount of salt generally added to butter should control *Ps. putrefaciens*, provided the salt is adequately distributed. However, as pointed out by Rahn (6), it is probable that not all water droplets in butter have the same salt content, since salt is added after washing when many small droplets are already enclosed in fat. Numerous lots of commercial butter with relatively high salt contents have developed the putrid condition. Claydon and Hammer (1) found that salt was not completely effective in controlling the putrid defect unless combined with thorough working. Probably factors other than salt distribution and working also are involved.

VIABILITY IN BUTTER

The viability of *Ps. putrefaciens* in butter was investigated with two lots, each including a salted and an unsalted portion, held at 21° and 3°C. Both lots were made from pasteurized cream, one being inoculated with a pure culture of *Ps. putrefaciens* and the other with the pure culture plus a mixture of organisms from a sample of putrid butter. The butter was examined for *Ps. putrefaciens* at the time of manufacture and at intervals thereafter by smearing serum on the special gelatin agar and also by adding serum to litmus milk for enrichment at 3°C.

In the unsalted butter the putrid defect developed rapidly at both 21° and 3°C. At 21°C. the numbers of *Ps. putrefaciens* after 24 hours were much larger than originally, and no reduction could be detected after 18 days; at 3°C. the numbers had greatly increased after 1 week, and there was no decrease after 16 weeks.

In the salted butter the 2.5 percent salt and the working evidently prevented the development of a definite putrid condition. However, at 21°C. there was an increase in numbers of *Ps. putrefaciens* after 24 hours; later there was a definite decrease. At 3°C. there was a slight increase in numbers up to 8 weeks and then a decrease.

VARIATION IN *Ps. putrefaciens*

In all, 176 cultures of *Ps. putrefaciens* were studied in more or less detail; 46 of them were from the collections of Derby and Hammer (3), Claydon and Hammer (1) and Shadwick (7), and 130 were recent isolations from various sources.

In some respects the cultures were very homogeneous. Each of the 176 cultures produced the putrid condition in skimmilk, and each of those tested produced it in butter, although there was some variation in time required and in degree of

the defect. When grown in sterile skimmilk, all the cultures examined produced phosphatase in relatively large amounts; this character appears important from the standpoint of identification. In addition, all the cultures rapidly reduced litmus milk, reduced nitrates to nitrites, formed large amounts of hydrogen sulfide and also ammonia. Variations in resistance to heat, acid and salt were negligible.

In certain other reactions the cultures showed considerable variation. Milk was proteolyzed by most cultures, some of them completing proteolysis in several weeks at 21°C., while others produced only partial proteolysis even on much longer incubation. A few cultures never showed visible proteolysis, and after several weeks some of these were acid and coagulated. A number of cultures produced ropiness in skimmilk; it was more pronounced and occurred more frequently at 3°C. than at 21°C., with certain cultures showing it at 3°C. but not at 21°C.

The extent of gelatin liquefaction at 21°C. was quite variable; this is in agreement with the results of Cullity and Griffin (2). Certain of the cultures completed liquefaction in about 16 days, while others did not complete it in much longer time. Although all the cultures produced definite liquefaction, with a few it was very slight, and these cultures were the ones that gave the least proteolysis in litmus milk.

At 21°C. on the special gelatin agar, the cultures varied in ability to initiate growth, in extent of growth and in color production. Apparently, it was much more difficult for some cultures than for others to establish growth on the medium, and this would be expected on the basis of experience with other media. Considerable variation occurred in colony size; some cultures regularly produced colonies only a few millimeters in diameter, whereas others produced colonies up to 8 mm. in diameter. The color on the special medium was variable, being distinctly brown with a few strains, whereas with the majority it was either reddish brown or pink.

As would be expected, there was a variation in the fermenting powers of the different cultures, some producing no change in the bouillons employed and others producing acid but no gas in certain of them. Acid was produced from lactose by a few cultures, and although there was not perfect correlation between fermentation of lactose in bouillon and acid production in litmus milk, there was a general relationship. Because of the large ammonia production by the organism, it is possible that with certain cultures acid is neutralized as rapidly as it is formed.

The rather wide variation between cultures is in agreement with previous results (1, 3); however, none of the variations appears sufficiently important to justify varietal designations. Un-

doubtedly the amount of growth is a factor in variation and must be considered. Some cultures are difficult to cultivate on artificial media, and even when growth is once established they do not develop as extensively as others; as a consequence, the changes produced in various media may differ from those produced by vigorous cultures.

GENERAL DESCRIPTION OF *Ps. putrefaciens*

MORPHOLOGY (CULTURES GROWN AT 21°C.)

FORM AND SIZE: Rods; 0.5 to 0.75 by 0.6 to 5.5 microns (averaging about 0.55 by 2.1 microns) when grown 1 to 2 days on agar.

ARRANGEMENT: Singly, in pairs and short chains.

STAINING REACTIONS: Stains readily with common stains; gram negative.

SPORES: None observed; the organism is easily destroyed by heat.

MOTILITY: Motile; commonly by means of a polar flagellum, although in a few cultures several polar flagella may be noted.

CULTURAL CHARACTERISTICS (CULTURES GROWN AT 21°C. WITH HEAVY INOCULATIONS)

AGAR SLANT: On beef infusion agar most cultures gave fair growth in 1 day and good growth in 2 days; growth usually raised, smooth, shiny, granular, colorless to grayish green, spreading and opaque; pronounced ammoniacal odor but little or no putrid odor; on extended incubation, color usually slightly brown or reddish brown. On special gelatin agar growth good in 1 day and heavy in 2 days; growth raised, smooth, granular, somewhat spreading and opaque in young cultures, becoming less granular and more opaque with age; color conspicuous in 2 days, being brown to reddish brown or pink; marked ammoniacal and somewhat putrid odor.

AGAR STAB: Heavy surface growth on beef infusion and special gelatin agars with much less growth along the line of inoculation.

AGAR COLONY (SMEAR TECHNIC): On beef infusion agar a convex, glistening, smooth, translucent, usually granular, colorless to grayish colony after 1 to 2 days, becoming brown to reddish brown on extended incubation; most cultures butyrous, some viscid; colony size 2 to 4 mm. with some cultures and 6 to 8 mm. with others. On special gelatin agar growth more vigorous than on beef infusion agar, but colony characters much the same with the exception of color,

the color being brown to reddish brown or pink in 2 to 3 days. Conspicuous ammoniacal odor with either medium; in addition, somewhat putrid odor on the special gelatin agar.

GELATIN STAB: Crateriform liquefaction, becoming infundibuliform on extended incubation. Rate of liquefaction varies considerably with different cultures, some completing it in 16 days, others not completing it on long incubation. Marked putrefactive odor in 7 to 10 days.

BEEF EXTRACT BROTH: Turbidity, sediment and a thin pellicle.

POTATO: Poor to moderate, spreading growth with irregular margin in 2 to 3 days; conspicuous reddish brown color.

LITMUS MILK: Litmus milk rapidly reduced, usually within 6 to 8 hours, by active cultures. Following reduction, most cultures proteolyze the milk, beginning at the surface and working downward until almost complete digestion occurs and a yellow to yellowish brown serum remains; digestion generally requires several weeks to a month for completion. Some cultures digest milk only slightly, and a few show no visible digestion, the latter sometimes producing coagulation and acid. Commonly a pellicle, which often is distinctly reddish brown, after several days. Ropiness produced by many cultures, being more pronounced at lower temperatures than at 21°C. In young milk cultures a distinct putrid odor while in older cultures a strongly objectionable, but less putrefactive, odor.

BIOCHEMICAL FEATURES (CULTURES GROWN AT 21°C. WITH HEAVY INOCULATIONS).

INDOL: Not produced.

NITRATES: Rapidly reduced to nitrites.

HYDROGEN SULFIDE: On peptone iron medium excellent growth, both in broth and on agar, with rapid production of H₂S. Objectionable odor.

VOLATILE ACID: Small amounts produced by a few cultures.

CARBON DIOXIDE: Produced in relatively large amounts.

METHYL RED REACTION: Negative.

VOGES PROSKAUER REACTION: Negative.

AMMONIA: Rapidly produced from peptone.

FERMENTING POWER: Considerable variation in the fermenting ability of various cultures; some produce no change in any of the various bouillons,³ while others produce acid but no gas from the following substances in order of frequency of attack: Maltose, sucrose, arabinose, dextrose, galactose, lac-

³ In some cases ammonia production by the organism may neutralize acid as rapidly as it is formed.

tose and levulose, the last two substances being only very rarely attacked. Neither acid nor gas produced from glycerol, inulin, mannitol, raffinose, salicin or soluble starch. Usually a culture producing acid from a carbohydrate later reverses the reaction.

LIPOLYSIS: Natural fats not hydrolyzed.

PHOSPHATASE: Rapidly produced in milk and other media; decreases to some extent on aging the culture.

pH: With 11 representative strains the pH values in milk after 32 days ranged from 5.7 to 7.0 and averaged 6.3.

GROWTH CONDITIONS

OXYGEN RELATIONSHIP: Facultative; grows well aerobically.

GROWTH TEMPERATURES: Grows at 30°C., 3°C. and temperatures in between. No growth at 37°C.

HEAT RESISTANCE: Heat resistance low, none of the cultures tested surviving 2 minutes at 61.7°C.

ACID RESISTANCE: Sensitive to acid; cultures commonly destroyed at pH values approximating 5.3 or lower.

SALT RESISTANCE: All cultures tested grew in litmus milk containing 4 percent salt and none in milk containing 10 percent; with 6 or 8 percent, cultures were variable.

IDENTIFICATION PROCEDURE

Colonies (suitably purified) that show the proper characters on the special gelatin agar are inoculated into litmus milk. If the typical reaction is produced, and the morphology of the cells is correct, the milk culture is tested for phosphatase. If positive, the organism is studied for its ability to produce the putrid defect in butter. Any additional tests that seem advisable are then carried out.

DISCUSSION OF RESULTS

The wide distribution of *Ps. putrefaciens* in water and perhaps in moist soil probably accounts for its presence in raw sweet milk and cream. Presumably it is brought into dairy plants of various types and then is present on floors and in equipment. In such equipment as churns, which are difficult to sterilize because various organisms penetrate cracks, joints, etc., *Ps. putrefaciens* may become established and infect one lot of butter after another. The large numbers of *Ps. putrefaciens* encountered in certain churns suggest extensive multiplication of the organism and account for heavy contamination of repeated churnings.

The finding of *Ps. putrefaciens* in butter which did not show the putrid defect and did not develop it on holding emphasizes the importance of growth in butter. Undoubtedly the organism is inhibited in many lots of butter. With salted butter, the salt concentration and distribution (as the result of working) presumably are the important inhibiting factors, and in highly ripened, unsalted butter, the acidity developed in the cream is of significance, but in both types of butter the influence of holding temperature must be recognized.

The low resistance of *Ps. putrefaciens* to heat suggests that it never survives proper pasteurization. In the case of cream having considerable acid, it is probable that any *Ps. putrefaciens* organisms originally present have been destroyed. These general relationships emphasize water and plant equipment as of primary significance in explaining outbreaks of putrid butter.

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